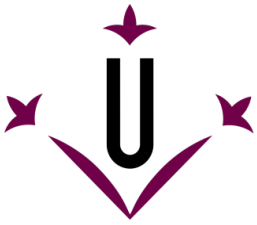

Plasma concentrations of pregnancy-associated glycoproteins I and II and progesterone on day 28 post-AI as markers of twin pregnancy in dairy cattle

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Abstract

Carrying twins is the main factor jeopardizing pregnancy maintenance and reducing the lifespan of dairy cows. There is therefore a need to accurately detect twin embryos at the time of pregnancy diagnosis so that twin pregnancies are carefully followed. This study compares in single and twin pregnancies from day 28 to 42 of gestation, patterns of plasma pregnancy-associated glycoproteins (PAGs) I and II and progesterone (P4), and establishes cut-offs for these hormones on day 28 post-AI that might be useful for twin pregnancy diagnosis. The sensitivity and specificity of the diagnostic capacity of these cut-off values were then determined using ultrasonography findings as the reference standard. A total of 110 lactating dairy cows carrying live singletons or twins from day 28 to 42 of gestation were enrolled. On day 28 post AI, 56 cows were diagnosed as carrying twins and 54 singletons by ultrasonography. This diagnosis was confirmed ultrasonographically on days 35 and 42 post AI. Among the 54 cows with single pregnancies, those in which the single embryo was accompanied by two corpora lutea (n=23) maintained this condition until day 42 and showed similar plasma PAG-I, PAG-II and P4 levels than cows with a single embryo and single corpus luteum (n=31). Cows bearing twins showed higher plasma PAG-I, PAG-II and P4 concentrations throughout the study period than cows bearing singletons (between subject effect; $P < 0.001$). Cutoffs for plasma PAG-I, PAG-II and P4 concentrations on day 28 of gestation of 5.5, 0.25 and 14 ng/ml, respectively, were used to determine twin pregnancies. Each of the proposed cut-off values identified twin pregnancies with an accuracy higher than 70%. In conclusion, the determination of these hormones could be a useful tool to discriminate between single and twin pregnancies.

Keywords: bovine, endocrinology, hormone, gestation, double ovulation

Introduction

The reduced fertility of modern high producing dairy herds makes the parturition-calving interval a key factor for herd survival. During the postpartum period, cows not only have to get pregnant but are also required to maintain their pregnancy until parturition. Pregnancy loss is effectively one of the most important factors for herd economy (Santos et al, 2004; López-Gatius, 2012).

Carrying twins has been extensively described as the main non-infectious factor compromising pregnancy maintenance during the late embryonic/early fetal period in dairy cows. The risk of pregnancy loss during the first trimester of gestation for cows carrying twins is three to nine times higher than for cows carrying singletons (López-Gatius and Garcia-Ispierto, 2010). Furthermore, twin birth is not desirable in dairy herds and income losses amount to a mean production lifespan 200 days shorter for cows calving twins than cows calving singletons (Andreu-Vázquez et al., 2012a). The twin pregnancy rate can exceed 18% in some herds (Andreu-Vázquez et al, 2012b) and, obviously, this incidence of twin pregnancies is closely related to the twin birth rate. Current estimates of the twin birth rate run at 9% and even exceed 12% in some herds (Silva del Rio et al., 2007). Thus, pregnancy diagnosis should include the detection of twin embryos to monitor these cows (López-Gatius and Garcia-Ispierto, 2010).

Pregnancy-associated glycoproteins I (PAG-I) and II (PAG-II) are members of the aspartic proteinase family that coexist in the ruminant trophectoderm (Garbayo et al., 2008; Wallace et al., 2015). Although the functions of these glycoproteins are not yet fully understood, plasma PAG-I concentrations have been used for pregnancy diagnosis

and as a marker of placental/fetal well-being (Zoli et al., 1992; Szenci et al., 2003; Wallace et al., 2015). Although studies have identified higher PAG-I plasma concentrations in twin pregnancies compared to single pregnancies (Patel et al., 1997; Echternkamp et al., 2006; López-Gatius et al., 2007), the diagnosis of twin pregnancy based on a PAG-I cut-off has recently been proven clinically insufficient before day85 of gestation (Szelényi et al.,2015). To the best of our knowledge, no study has examined both PAG-I and PAG-II patterns immediately following a positive pregnancy diagnosis in twin pregnancies.The present study was designed to compare plasma PAG-I, PAG-II and progesterone (P4) patterns from day28–42 of gestation between single and twin pregnancies and accordingly establish cut off-values for these hormones on day 28 post AI for twin pregnancy diagnosis. The sensitivity and specificity of the established cut-offs were determined using ultrasonography findings as the reference standard.

Materials and methods

Cattle and herd management

This study was performed on a commercial dairy herd of 1265 Holstein-Friesian lactating dairy cows in northeastern Spain from November 2014 to April 2015. Cows were milked three times daily with a mean annual milk production per cow of 11425 kg were fed complete rations. Only healthy cows free of detectable reproductive disorders and free of clinical diseases during the study period (days 28 to 42 of gestation) were included. Exclusion criteria were the disorders: mastitis, lameness, digestive disorders and pathological abnormalities of the reproductive tract and/or a fetus detectable on ultrasonography. These exclusions were made to reduce variation in the general health

state of the animals, so that plasma PAG and P4 changes could be attributed to factors not associated with these clinical conditions during the study.

Pregnancy diagnoses and experimental design

Pregnancy was diagnosed by ultrasonography on day 28 post AI during which numbers of embryos and corpora lutea were recorded. These numbers were revised on days 35 and 42 of gestation. The viability of the embryos/foetuses was confirmed by the observation of a heartbeat in all exams. The study population comprised 110 cows carrying live singletons or twins during the experimental period. For each twin pregnancy detected, a single pregnancy was selected. Cows were inseminated after 80 days in milk to avoid interference by residual PAG in the peripheral circulation during the postpartum period (Zoli et al., 1992). Cows were classified into 3 groups: cows with two embryos and two corpora lutea(n=56); cows with a single embryo and two corpora lutea, i.e., an additional corpus luteum (n=23); and cows with one embryo and its corresponding corpus luteum (n=31).

Blood sampling and PAGs and P4 determinations

Blood samples were withdrawn into heparinized tubes from the tail vein of each animal immediately after each ultrasonography examination. These blood samples were centrifuged (15 min at 1,500 x g) within 30 min of collection, and plasma was stored at –20ºC until assayed. All samples for each hormone were assayed simultaneously.

Plasma P4 concentrations were determined with a RIA-kit (Prog-RIA-CT ;product catalogue number KIP1458, DIAsource, Louvain-la-Neuve, Belgium). This test was performed according to the manufacturer's instructions and validated and described previously (López-Gatius et al., 2007). The minimum detection limit of the assay was 0.05 ng/ml. The intra-assay and inter-assay coefficients of variation were 7.3% (5.3± 0.4 ng/ml) and 12.9% (2.9± 0.4 ng/ml),respectively.

Plasma concentrations of PAG-1 measurements were determinate using a double antibody radioimmunoassay procedure (RIA-706). Rabbit polyclonal antiserum AS# 706 was raised against caprine PAG $_{55\ kDa+62\ kDa}$ (accession numbers P80935 and P80933) according to Vaitukaitis et al. (1971). The minimum detection limit for the RIA procedure was 0.2 ng/mL. Intra- and inter-assay coefficients of variation were 5.3% and 6%, respectively.

The bovine PAG-2 radioimmunoassay used has been recently described (Serrano et al., 2014). The primary antibody was rabbit polyclonal antiserum against boPAG-2 (AS#438) raised according to the method of Vaitukaitis (1971). The minimum detection limit calculated for RIA-438 was 0.12 ng/mL. Intra- and inter-assay coefficients were 4.6% and 4.8%, respectively.

Data collection analysis

The following data were recorded for each animal: parity (primiparous versus multiparous), days in milk, twin pregnancy (singletons versus twins), presence of an additional corpus luteum (two corpora lutea in cows carrying one single embryo), date

of AI, milk production at AI (≤40Kg versus > 40Kg), plasma concentrations of PAG-1 and PAG-2 and P4 on days 28 (1), 35 (2) and 42 (3) post AI. Insemination dates were used to assess the effects of season on subsequent reproductive performance. It should be noted that in our geographical region, there are only two clearly differentiated weather periods: warm (May to September) and cool (October to April) (Labernia et al., 1998; Garcia-Ispierto et al., 2007). The effects of the above variables on PAG-1 and PAG-2 and P4 of were evaluated by General Lineal Models (GLM) repeated measures analysis of variance.

Diagnosis were compared with ultrasound findings as follows: correct twin diagnosis (a); incorrect twin diagnosis (b); correct singleton diagnosis (c) and incorrect singleton diagnosis (d). From these values the sensitivity (100xa/a+d) and the specificity (100xc/c+b) of the pregnancy test were calculated.

The observation of one or two embryos proper with a beating heart was used as the criterion for positive singleton or twin diagnosis. To detect a twin pregnancy, cut-off values for plasma PAG-I, PAG-II and P4 concentrations on day 28 of gestation were established increasing 10% of the mean values for single pregnancies. Thus, 5.5, 0.25 and 14 ng/ml, respectively, were used to indicate a twin pregnancy. All the analyses were performed using the SPSS computer package, version 18.0 (SPSS Inc., Chicago, IL, USA). Significance was set at $p<0.05$.

Results

On day 28 post AI, 56 cows were diagnosed as carrying twins and 54 singletons by ultrasonography. This diagnosis was confirmed by ultrasound on days 35 and 42 post

AI. All cows carrying twins had two corpora lutea and embryos were positioned in the uterine horns ipsilateral to the side of the corpora lutea. Cows carrying singletons with an additional corpus luteum (n=23) maintained this condition until day 42.No significant differences were detected in plasma PAG-I, PAG-II and P4 concentrations shown by cows with a single or additional corpus luteum. Cows bearing twins (n=56) showed higher plasma PAG-1, PAG-2 and P4 concentrations throughout the study period than cows bearing singletons (n=54) (between subject effect; $P < 0.001$) (Fig. 1). Parity, days in milk, milk production at AI, presence of an additional corpus luteum and season of AI had not effect on plasma PAG-I, PAG-II and P4 values.

Table 1 shows the sensitivity and specificity of the use of plasma PAG-1, PAG-2 and P4 concentrations on day 28 of gestation as markers of twin pregnancy. Each of the proposed cut-off values served to identify twin pregnancies with precision greater than 70%.

Discussion

In this study, the sensitivity and specificity of plasma PAG-1, PAG-2 or P4 values to detect twin pregnancies on day 28 of gestation proved valuable at the herd level. As far as we know, no prior study has determined cut-off values for PAGs and P4 for twin pregnancy diagnosis in dairy cows. Although the sensitivity and specificity of these markers was not 100%, the use of either marker or their different combinations could be an option in herds for which ultrasonography is unavailable. In effect, from a clinical point of view, detection of at twin pregnancy as early as possible is very helpful within control reproductive programs. For example, GnRH treatment given to cows carrying

twins at pregnancy diagnosis can favour maintenance of gestation (López-Gatius and Garcia-Ispierto, 2010)

The PAG-1 assay has been proposed as an alternative to ultrasonography provided it is f used at proper time after calving (80 days postpartum) (Zoli et al., 1992). Although Szelényi et al. (2015) were able to select a cut-off value of 39.4 ng/ml to discriminate between singleton and twin pregnancies using the ELISA test,the sensitivity of their method was as low as 28.6%.In our study, the sensitivity of the PAG-I test was 76%. This large difference may be attributed to the different dates of ultrasonographic pregnancy diagnosis, from gestation days 29–42 in the study by Szelényi et al. (2015). Plasma PAG-I concentrations show a sigmoid shaped growth curve (Green et al.,2005) and this is likely to hinder comparisons between single and twin pregnancies.

The recent detection of PAG-II molecules in blood has paved the way for investigations on placental well-being and pregnancy loss in cows. In effect, PAG-2 plasma have been proposed as an indicator of abortion risk in *Neospora caninum*-infected cows (Garcia-Ispierto et al, 2013). In the present study, PAG-II concentrations were able to distinguish between singletons from twins at the time of pregnancy diagnosis. Although sensitivity and specificity were lower than for PAG-1, this finding unveils a wide range of analytical possibilities with clinical implications.

Conclusions

Elevated plasma PAG-I, PAG-II and P4 concentrations detected on day 28 post AI in dairy cows carrying twins determines that these hormones are useful markers to discriminate between single and twin pregnancies.

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References

Andreu-Vázquez C, Garcia-Ispierto I, Ganau S, Fricke PM and López-Gatius F 2012c. Effects of twinning on the subsequent reproductive performance and productive lifespan of high-producing dairy cows. Theriogenology 78:2061-2070.

Andreu-Vázquez C, Garcia-Ispierto I and López-Gatius F 2012b. Photoperiod length and the estrus synchronization protocol used before AI affect the twin pregnancy rate in dairy cattle. Theriogenology 78:1209-1216.
cows. Journal of the American Veterinary Medical Association 231, 1390–1397.

Echternkamp, S.E., Vonnahme, K.A., Green J.A., Ford, S.P., 2006.Increased vascular endothelial growth factor and pregnancy associated glycoproteins, but not insulin-like growth factor-I, in maternal blood of cows gestating twin fetuses. J. Anim. Sci. 84, 2057–2064.

Garbayo JM, Serrano B and López-Gatius F 2008. Identification of novel pregnancy-associated glycoproteins (PAG) expressed by the peri-implantation conceptus of domestic ruminants. Animal Reproduction science 103, 120-134.

García-Ispierto I, López-Gatius F, Santolaria P, Yániz JL, Nogareda C, López-Béjar M 2007. Factors affecting the fertility of high producing dairy herds in northeastern Spain. Theriogenology 67, 632-638.

García-Ispierto I, Almería S, Serrano B, de Sousa NM, Beckers JF and López-Gatius F 2013. Plasma concentrations of pregnancy-associated glycoproteins measured using anti-bovine PAG-2antibodies on day 120 of gestation predict abortion in dairy cows naturally infected with Neospora caninum. Reproduction in Domestic Animals 48, 613-618.

Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, McMillan W, Mathialagan N, Hook RR, Xie S and Roberts RM 2005. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 63, 1481-1503.

López-Gatius, F., Garbayo, J.M., Santolaria, P., Yániz,J ., Ayad, A., deSousa, N.M., Beckers, J.F., 2007.Milk production correlates negatively with plasma concentrations of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. Domest. Anim. Endocrinol. 32, 29–42.

López-Gatius F and García-Ispierto I 2010. Ultrasound and endocrine findings that help to assess the risk of late embryo/early foetal loss by non-infectious cause in dairy cattle. Reprod. Domes. Anim. 45 (Suppl 3), 15-24.

Código de campo cambiado

Código de campo cambiado

López-Gatius F, 2012. Factors of a non infectious nature affecting fertility after artificial insemination in lactating dairy cows.A review.Theriogenology 77, 1029–1041.

Patel OV, Sulon J, Beckers JF, Takahashi T, Hirako M, Saski N 1997. Plasma bovine pregnancy-associated glycoprotein concentrations throughout gestation in relationship to fetal number in the cow. European Journal of Endocrinology 137, 423-428.

Santos JEP, Thatcher WW, Chebel RC, Cerri RLA and Galvão KN 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Animal Reproduction Science 82-83:513-535.

Serrano-Pérez B, Garcia-Ispierto I, de Sousa N, Beckers J, Almería S, López-Gatius F 2014. Gamma interferon production and plasma concentrations of pregnancy-associated glycoproteins 1 and 2 in gestating dairy cows naturally infected with Neospora caninum. Reproduction in Domestic Animals 49, 275-280.

Silva del Rio N, Stewart S, Rapnicki P, Chang YM and Fricke PM 2007. An observational analysis of twinbirths, calf sex ratio, and calf mortality in Holstein dairy cattle. Journal of Dairy Science 90, 1255-1264.

Szelényi Z, Répási A, de Sousa, Beckers JF and Szenci, O. 2015. Accuracy of diagnosing double corpora lutea and twin pregnancy by measuring serum progesterone and bovine pregnancy-associated glycoprotein 1 in the first trimester of gestation in dairy cows. Theriogenology 84, 76-81.

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Szenci, O., Beckers, J.F., Sulon, J., Bevers, M.M., Börzsönyi, L., Fodor, L.,Kovács, F., Taverne, M.A.M., 2003. Effect of induction of late embryonic mortality on plasma profiles of pregnancy associated glycoproteins in heifers. Vet. J. 165, 307–313.

Vaitukaitis J, Robbins JB, Nieschlag E, Ross GT 1971. A method for producing specific antisera with small doses of immunogen. Journal of Clinical Endocrinology and Metabolism 33, 988–991.

Wallace RM, Pohler KG, Smith MF and Green JA 2015. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction 149, R115-R126.

Zoli AP, Guibault LA, Delahaut P, Benitez Ortiz W and Beckers JF 1992. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. Biology of Reproduction 46, 83–92.

Tables

Table 1. Sensitivity and specificity of plasma PAG-1, PAG-2 and P4 concentrations for the diagnosis of single or twin pregnancy. The reference diagnostic criterion was the observation of one or two embryos with a beating heart by ultrasonography.

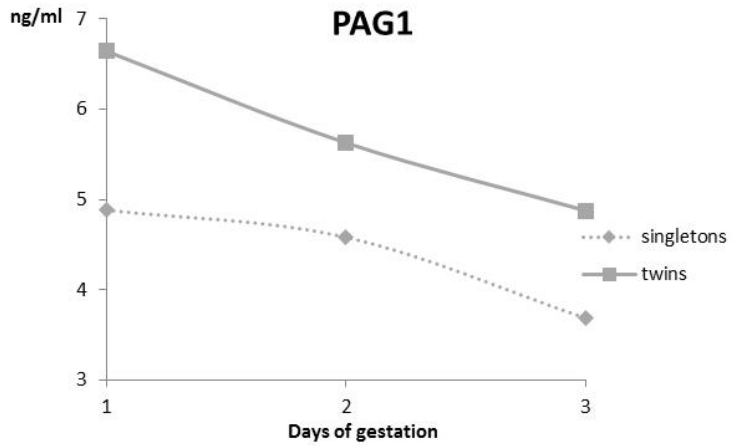
Hormone	Sensitivity (%)	Specificity (%)
PAG1 (5.5 ng/ml)	76	80
PAG2 (0.25 ng/ml)	73.2	74
P4 (14 ng/ml)	62.3	84

Sensitivity: lOO x a/a+d; specificity: lOO x c/c+b; where a = correct twin diagnosis, b = incorrect twin diagnosis, c = correct singleton diagnosis, and d = and incorrect singleton diagnosis.

1 Figure Captions

2 Figure 1. Mean (± S.E.) plasma PAG-1 (A), PAG-2 (B) and P4 (C) concentrations
3 recorded on Day 28 (1), 35 (2) and 42 (3) post AI in cows bearing singletons (n=54) or
4 twins (n=56). All values differed significantly between twin and single pregnancies
5 (between subject effect repeated measures ANOVA; $P<0.001$).

Figure 1.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Days of gestation
singletons
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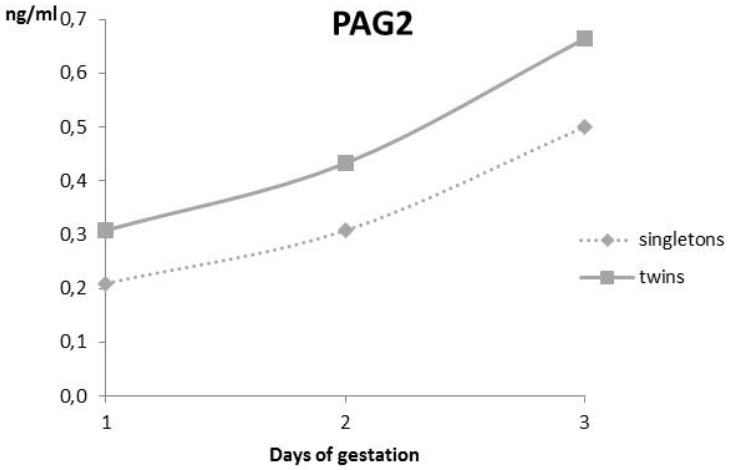
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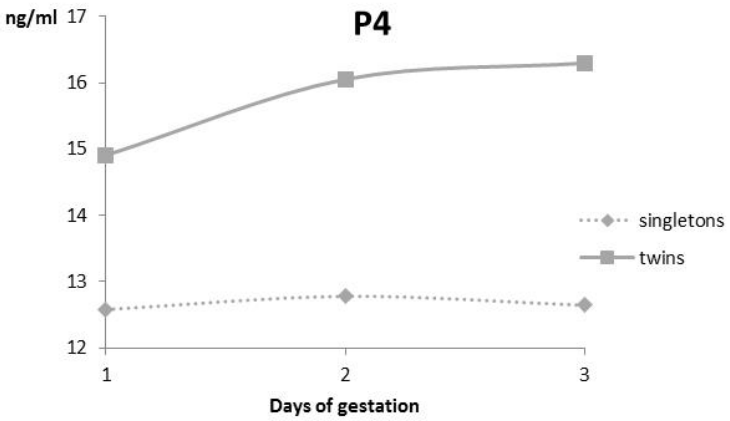
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Days of gestation
singletons
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